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Collection of highly germinative pseudochain conidia of *Oidium neolycopersici* from conidiophores by electrostatic attraction

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ABSTRACT

A population of simultaneously germinating conidia is an ideal inoculum of the powdery mildew pathogen, *Oidium neolycopersici*. In conditions of no or low wind velocity, *O. neolycopersici* successively stacks mature conidia on conidiophores in a chain formation (pseudochain), without releasing the precedent mature conidia. These pseudochain conidia represent a perfect inoculum, in which all conidia used for inoculation germinate simultaneously. However, we found that conidia must be collected before they fall to the leaf surface, because the germination rate was lower among conidia deposited on the leaf surface. We used an electrostatic spore collector to collect the pseudochain conidia, and their high germination rate was not affected by this treatment. The spore collector consisted of an electrified insulator probe, which created an electrostatic field around its pointed tip, and attracted conidia within its electric field. The attractive force created by the probe tip was directly proportional to voltage, and was inversely proportional to the distance between the tip and a target colony on a leaf. Pseudochain conidia were successfully collected by bringing the electrified probe tip close to target colonies on leaves. In this way, conidia were collected from colonies at 3-d intervals. This effectively collected all conidia from conidiophores before they dropped to the leaf surface. A high germination rate was observed among conidia attracted to the probe tip (95.5 ± 0.6 %). Conidia were easily suspended in water with added surfactant, and retained their germination ability. These conidia were infective and produced conidia in pseudochains on conidiophores after inoculation. The electrostatic spore collection method can be used to collect conidia as they form on conidiophores, thus obtaining an inoculum population in which all of the conidia germinate simultaneously.

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Introduction

Oidium neolycopersici is a powdery mildew pathogen of tomato. It has been reported worldwide (Kiss *et al.* 2001) and also in Japan (Kashimoto *et al.* 2003a). The Japanese isolate of *O. neolycopersici*, KTP-01, is highly pathogenic to all commercial cultivars tested (Matsuda *et al.* 2001; Kashimoto *et al.* 2003a), causing serious agricultural problems in tomato crop production throughout Japan (Shimizu *et al.* 2007). The pathogen produces conidia on conidiophores singly or in pseudochains of two to four mature conidia (Kashimoto *et al.* 2003a). Our observations of conidiophores on tomato leaves revealed that the pathogen released mature conidia from the apical tip of conidiophores at 20-h intervals (Oichi *et al.* 2004) at wind velocities of 0.8 to 1.2 m s^{-1}, but they did not release mature conidia under conditions of no or low wind velocity (Oichi *et al.* 2006). KTP-01 elongates pseudochains consisting of a maximum of four mature conidia (Oichi *et al.* 2006). The wind speed in the greenhouse was insufficient to blow conidia from the conidiophores, thus the longest pseudochains frequently fell to the leaf surface (Oichi *et al.* 2006). As a result, the infected tomato plants carry abundant conidia on leaves, which are thought to be a possible source of infection, leading to further dissemination of the disease (Whipps *et al.* 1998; Mieslerová & Lebeda 1999; Jones *et al.* 2001). These infected leaves have been used as inoculum, by directly dusting the conidia onto test leaves with a paintbrush or spraying them in a suspension. These are conventional inoculation methods for powdery mildew pathogens (Nicot *et al.* 2002). However, it is not obvious whether the germination rates of conidia on leaf surfaces and those on conidiophores are equally high, nor how long they remain germinative. This information is essential to obtain an ideal inoculum population in which conidia have a high germination rate. We aimed to compare germination rates between conidia in pseudochains and those accumulating on the leaves using conidia collected separately from each source. We expected that an electrostatic method to attract conidia would be a useful tool for this purpose.

An insulator can be electrified through dielectric polarization caused by a charged conductor (Griffith 2004; Halliday *et al.* 2005). A polarized dipole insulator produces a non-uniform electric field around it, and creates an electrostatic force that attracts conidia within the electric field (Matsuda *et al.* 2006; Moriura *et al.* 2006a). Using this method, we constructed a spore precipitator to trap conidia released from the conidiophores of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Moriura *et al.* 2006b). This technology has also been applied to prevent pathogens (Matsuda *et al.* 2006; Shimizu *et al.* 2007) or insect pests (Tanaka *et al.* 2008) from entering the greenhouse. In addition, pathogen development on leaves could be consecutively traced with a high-fidelity digital microscope without detrimentally affecting fungal infection behaviour (Matsuda *et al.* 2005a; Oichi *et al.* 2004, 2006). We have also developed techniques to electrostatically manipulate conidia on leaves or on conidiophores in a microscopic field (Moriura *et al.* 2006a). In the present study, a newly developed portable spore collector was proposed to specifically obtain highly germinative conidia from pseudochains of *O. neolycopersici* colonies on tomato leaves. Conidia were maintained on young tomato seedlings under regulated conditions in a growth chamber, and the spore collector was used to obtain an ideal inoculum population in which all the conidia germinate simultaneously.

Materials and methods

Plant materials

Germinated seeds of *Lycopersicon esculentum*, cv. 'Moneymaker' were sown in vermiculite in a tray and grown in a growth chamber for 7 d in the following environmental conditions: 25 ± 0.5 °C; 95–100 % RH; and continuous illumination of 3500 lux provided by fluorescent lamps. Once the cotyledon leaves had unfolded, plants were transplanted to soil in 15-cm pots and grown in a temperature-controlled greenhouse (25 ± 3 °C) for one month. These one-month-old seedlings were used for inoculation experiments.

Pathogen and inoculation

Conidia of *Oidium neolycopersici* (KTP-01) (Kashimoto *et al.* 2003a) were used in the present study. To maintain KTP-01, conidia of KTP-01 formed on inoculated leaves were dusted onto leaves of fresh two-month-old tomato seedlings (cv. 'Moneymaker') with a paintbrush with soft bristles (dusting inoculation method) every two weeks. Moneymaker is highly susceptible to KTP-01 (Matsuda *et al.* 2005b). The powdery mildew colonies that formed on the leaves of these inoculated plants were used to inoculate test plants. Voucher material (KUH-ONL1) of the fungus used is preserved in Herbarium Preservation Section of Kinki University.

Conidia of 10-d-old colonies were inoculated onto leaves of one-month-old seedlings by the dusting inoculation method. Inoculated plants were transferred to the growth chamber (the same conditions mentioned earlier). Formation of conidiophores and conidial pseudochains on the inoculated leaves was observed without detaching leaves using a high-fidelity digital microscope KH-2700 (Hirox, Tokyo) according to the method described previously (Moriura *et al.* 2006a). Under these conditions, powdery mildew pustules became visible on leaves 4 d after inoculation; the pustules at this stage were designated as 1-d-old colonies. Conidia were collected using the electrostatic attraction method from powdery mildew colonies at several time intervals after inoculation, and their germination ability and infectivity was determined.

Portable spore collector

The polarized dielectric insulator (ebonite) probe was originally constructed to collect conidia produced by barley powdery mildew (Moriura *et al.* 2006b). This probe was refined as a new portable spore collector. The insulator probe (6 mm diam; 20 mm length) with a pointed tip (tip diameter 0.2 mm) was connected with a conductor (copper) ring in a transparent acrylic cylinder (insulator) at the flat end of the probe. The conductor ring was linked *via* an electric wire

to a battery-operated, portable, electrostatic voltage generator KMT-30 K (Max-Electronics, Tokyo; Fig 1A). The conductor ring was negatively charged to dielectrically polarize the insulator probe (for electrification of the probe); the negatively charged conductor ring polarizes the probe negatively on the pointed tip end surface and positively on the flat end surface (Moriura et al. 2006a). The static electricity at the pointed end of the probe was measured by touching the point to the probe of a coulometer NK-1001 (Kasuga Denki, Tokyo). The electricity level was controlled by changing the voltage supplied to the conductor ring. The voltage was measured with an electrostatic field meter FMX-002 (Simco, Kobe). The negative charge on the outer surface of the electrified probe tip generates an electrostatic field and creates an attractive force to trap conidia that come into this field (Matsuda et al. 2006; Moriura et al. 2006b).

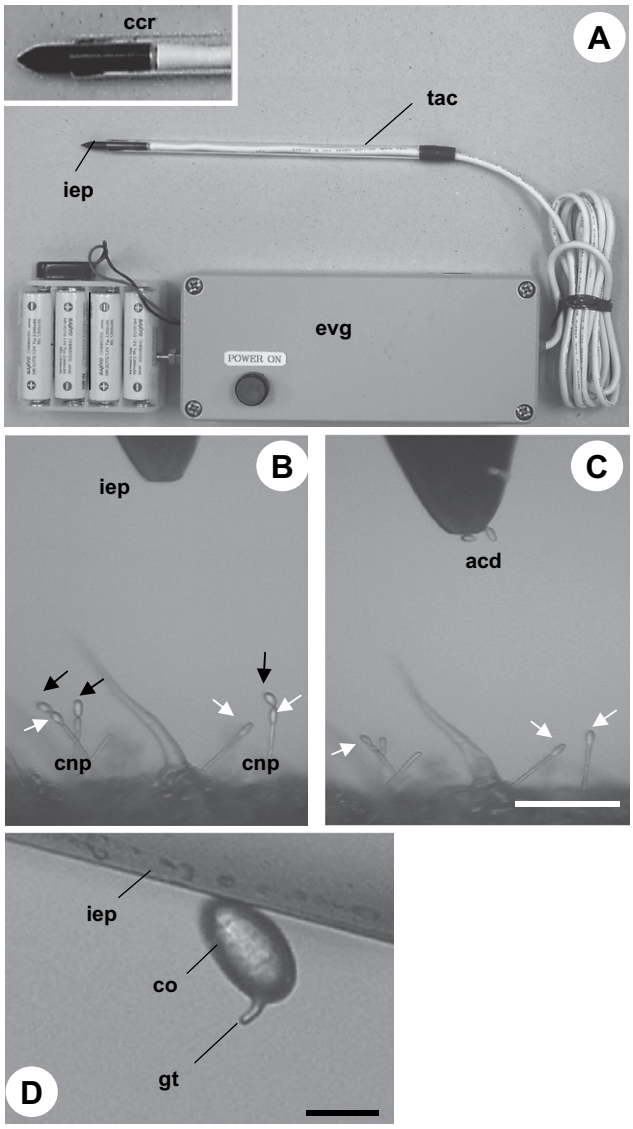

Fig 1 – Collection of *Oidium neolycopersici* pseudochain conidia from conidiophores on tomato leaves with an electrostatic spore collector. (A) Electrostatic spore collector. (B–C) Pseudochain conidia (B) on conidiophores before attraction to the probe and (C) electrostatically attracted to the probe tip. Conidia were attracted when the probe tip was positioned 100 μm from conidia on conidiophores. Black and white arrows represent mature and immature conidia on pseudochain conidiophores, respectively. (D) Conidia germinating on the detached probe tip during incubation. Abbreviations: iep, insulator ebonite probe; tac, transparent acrylic cylinder; ccr, conductor copper ring; evg, electrostatic voltage generator, cnp, conidiophore; acd, attracted conidia; co, conidium; gt, germ tube. Bars = (C) 50 μm, (D) 10 μm.

Collection of conidia

The insulator probe was held by a manipulator on the digital microscope, and various levels of static electricity were supplied to the probe tip surface. The relationship between the level of electricity and the ability to attract conidia to the probe tip was examined under the digital microscope. In this experiment, the electricity level of the tip surface was 5–6 $\times 10^{-1}$ nC under an electrostatic voltage of 0.5–30 kV. The probe tip was moved slowly towards colonies on leaves while viewing the attraction of conidia under the digital microscope. The distance at which pseudochain-forming conidia on conidiophores were first attracted to the electrified probe tip was recorded to quantify the attractive force.

To collect pseudochain conidia, the probe was moved over colonies on inoculated leaves two or three times at a distance of 100 μm, at a setting of 5 kV, while confirming the attraction of conidia on conidiophores under the digital microscope. The number of conidiophores in the colony was counted under the digital microscope after the conidia were removed. Pseudochain conidia were consecutively collected from the same powdery mildew colonies every 3 d; the first collection was conducted when target colonies were 5-d-old and the last when colonies were 20-d-old.

After the final collection of conidia, detached leaves were decoloured in a boiling alcoholic lactophenol solution for 1–2 min and stained with aniline blue. Stained colonies were photographed to determine the area of colonies with a charge-coupled device (CCD) camera of the digital microscope. The area was estimated using Scion image-analysis computer software (Scion, Frederick, MD). Ten colonies on different leaves were used for each collection of conidia. Data are shown as means and standard deviations of three replications.

Germination and infectivity assays

We first determined whether conidia collected electrostatically retained a high germination rate. To do this, the conidia attracted to the probe tip were counted under the digital microscope. The probe was then detached from the collector and incubated for 10 h under optimal germination conditions for KTP-01 conidia: 25 ± 0.5 °C; 95–100 % RH; and continuous illumination of 3500 lux (Kashimoto et al. 2003a). At the same time, conidia from colonies were directly dusted onto an ebonite plate with a paintbrush and their germination rate was similarly examined. In this experiment, different colonies on different leaves were used for the dusting, as brushing

colonies damaged conidiophores, affecting subsequent production of progeny conidia.

In the second experiment, the probe tip was dipped into water to prepare a suspension of electrostatically attracted conidia. Surfactant was added to water to rapidly suspend the conidia. We examined toxicity of surfactants to the conidia prior to use. Five commercial spreaders were used in this experiment: Mixpower and Submerge (Syngenta Japan, Ibaraki), Silwet L-77 (OSi Specialities, Danbury, CT, USA), Dine (Sumitomo Chemical Garden Products, Tokyo), and Mairino (Nihon Nohyaku, Tokyo). Various dilutions of the conidia (between 10- and 10^5-fold dilutions) were prepared in distilled water. Conidia attracted to the probe tip were suspended in distilled water (1 ml) containing a surfactant by dipping the probe tip into the solution. The conidial suspensions were incubated at 25 °C, and aliquots were sampled at 5 and 30 min. Cellular disorganization of the suspended conidia was observed using LM.

To test germination and infectivity, the conidia attracted to the probe tip were suspended in water containing Mixpower (10^4-fold diluted), and 100 μl of the suspension (10^4 conidia ml^{-1}) was sprayed onto leaves of tomato seedlings. Inoculated plants were placed in the growth chamber. At various times after inoculation, inoculated leaves were observed with the digital microscope according to the method described previously (Matsuda *et al.* 2005a). We determined rates of conidial germination, appressorium formation, and haustorium formation (as determined by successful elongation of secondary hyphae). For comparative purposes, conidia deposited on the leaf surfaces were collected and their germination and infectivity were determined. Pseudochain conidia from all the conidiophores of a single powdery mildew colony were collected with the electrified probe, and then the colony was covered with 1 ml water containing a surfactant. The water was moved up and down several times with a pipette (pipetting method), and the conidial suspension was used for germination and infectivity assays.

Results

Under the conditions in this study, powdery mildew colonies were first visible on infected leaves 4 d after inoculation. At that point, 10 to 20 conidiophores per colony were erect from the superficial mycelia of the pathogen, but there were no mature conidia at their tips. Mature conidia first appeared on conidiophores 6 d after inoculation, and conidial pseudochains formed during the following 3 d. At 9 d post-inoculation, the colonies (6-d-old colonies) included six types of conidiophores (Fig 2). Mature conidia showed fully constricted septa between the conidial cells and the adjacent apical cells. From 9 to 10 d after inoculation, four-conidia pseudochains began to fall from conidiophores onto leaf surfaces. The conidiophores produced new conidial cells at their tips and produced pseudochains for the next 3 d. The powdery mildew conidiophores repeated pseudochain formation in this way. At the same time, new conidiophores developed in the areas of colony expansion. During a period of approximately three weeks, abundant pseudochain conidia fell from the conidiophores onto the leaf surfaces. In the following experiments,

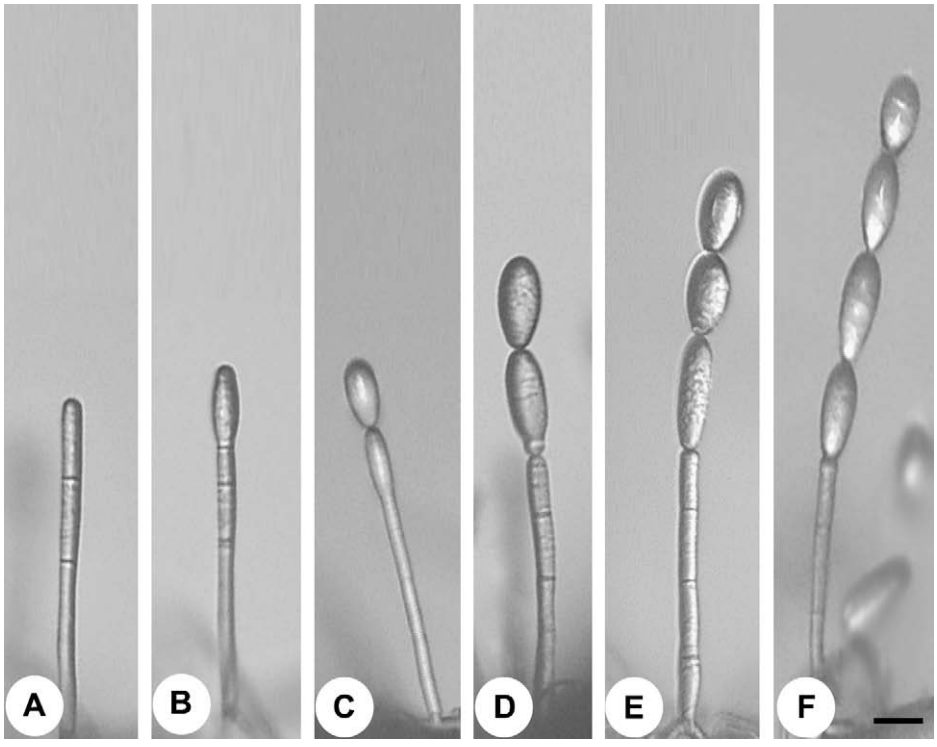

Fig 2 – Conidiophores of *Oidium neolycopersici* in 6-d-old colonies on tomato leaves. Colonies produced conidiophore with non-swollen apex (A) and conidiophores with a conidial cell developing at the tip (B), carrying a single mature conidium (C), forming pseudochains with two (D), three (E) and four mature conidia (F). Bar = 10 μm.

pseudochain conidia on conidiophores were electrostatically collected from the same colonies every 3 d (tridaily collection), beginning with 5-d-old colonies. This tridaily collection successfully collected all conidia before four had accumulated in conidiophores, the point at which pseudochains fall to the leaf surface.

The relationship between the voltage applied to the conductor ring and the electrical force at the probe tip surface is shown in Fig 3A, and the relationship between the electrical force at the probe tip and the attractive force is shown in Fig 3B. The charge at the probe tip surface was directly proportional to the voltage on the conductor ring, and the electrostatic force to attract conidia varied with respect to distance from the leaves. In the subsequent experiments, conidial collection was conducted by negatively charging the conductor ring with 5 kV and by positioning the probe tip 100 μm from the sample material.

We demonstrated successful attraction of pseudochain conidia from conidiophores of 5-d-old colonies on inoculated leaves (Fig 1B–C). A high germination rate was observed among conidia attracted to the probe (Fig 1D). Almost all conidia germinated on the probe within 10 h under the present incubation conditions (Fig 4A). High germination rates were also observed among the pseudochain conidia produced successively by the conidiophores from which mature conidia were removed electrostatically (Fig 4A). These results indicate that conidiophores produce highly germinative conidia through repeated cycles of pseudochain production, and the conidia in pseudochains retain their germination ability even after they were collected electrostatically. The germination rate of conidia dusted from the colonies was lower than that of pseudochain conidia (Fig 4B). This lower germination rate was statistically significant.

It was possible to collect almost all pseudochain conidia from conidiophores in target colonies with the electrostatic spore collector. Fig 5 shows the number of conidia collected from the same single colonies on inoculated leaves at 3-d-intervals during the entire period of pathogen pseudochain formation. The number of conidiophores per colony is also plotted in this figure. The development of conidiophores ceased at 17 d after inoculation. The number of the attracted conidia increased gradually with continuous development of new conidiophores, reaching a maximum 17 d after inoculation (14-d-old colonies) and was markedly lower 20 d after inoculation (17-d-old colonies). The difference was statistically significant. The colony area was $0.3 \pm 0.02\,cm^2$ at the time of the final collection of conidia (23 d after inoculation).

Addition of surfactants effectively suspended the attracted conidia in water. However, four of the commercial spreaders (Silwet, Dine, Submerge, and Mairino) caused abnormal morphological changes soon after the conidia were suspended. Abnormal morphological changes included membrane disorganization, cytoplasmic granulation, and small vesicle formation in conidial cells. Silwet, Submerge, and Dine caused membrane disorganization and/or granulation, and Mairino caused granulation at these dilutions. Submerge caused cells to burst at lower dilutions (ten to 10^3-fold dilution). These detrimental effects were observed even when the spreaders were diluted 10^5-fold (Table 1). Conversely, Mixpower did not cause any visible morphological changes in conidia at 10^4-fold dilution, even after the conidia were suspended for 30 min, although small vesicles formed in conidial cells within 5 min at lower dilutions (Table 1). Mixpower at 10^4-fold dilution was useful to prepare conidial suspensions, and to spread the suspended conidia over the surface without forming water drops when the suspension was sprayed onto leaves of tomato seedlings.

We traced infection behaviours of conidia that were sprayed onto tomato leaves under the digital microscope (Table 2). Almost all conidia (92.8 ± 1.5 %) germinated on leaves within 10 h, but more than 50 % of the germinating conidia projected germ tubes upwards (Fig 6A). These conidia did not form appressoria throughout the entire period of incubation (48 h). Approximately 40 % of the inoculated conidia

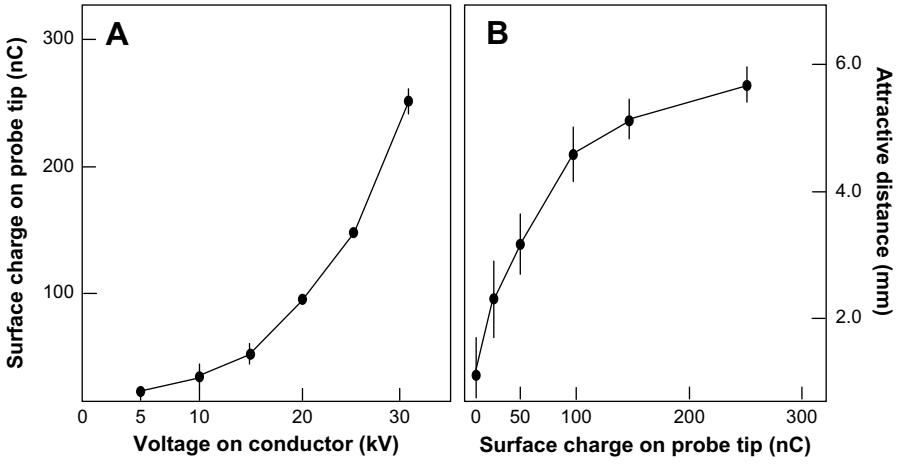

Fig 3 – Optimization of electrostatic conditions to attract pseudochain conidia from conidiophores of *Oidium neolycopersici* colonies on tomato leaves. (A) Relationship between electrostatic voltage supplied to conductor ring and static electricity at probe tip surface. (B) Relationship between electricity and attraction at tip surface of insulator probe. Each electricity level was tested on 20 colonies on different leaves. Data are means and standard deviations of five replications. Standard deviations are presented by error bars on the graphs.

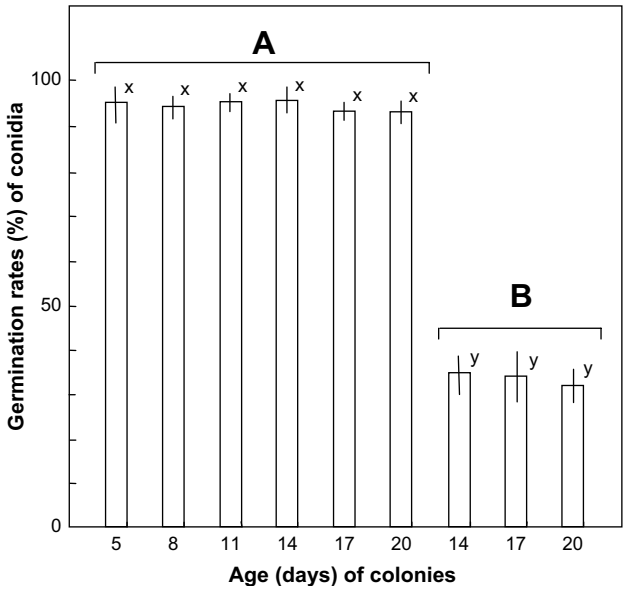

Fig 4 – Germination of conidia collected from colonies of *Oidium neolycopersici* KTP-01 on tomato leaves. (A) Pseudochain conidia on conidiophores were electrostatically collected from the same colonies every 3 d using the electrified probe (−5 kV) and incubated for 6 h to determine their germination rate on the probe. (B) Conidia from both pseudochains and leaf surface were dusted onto an ebonite plate with a paintbrush and incubated for 6 h to determine their germination rate. Different colonies on different leaves were used for each dusting. Ten colonies on different leaves were used for each collection, and data are means and standard deviations of five replications. Standard deviations are represented by error bars on the graph. Different letters on mean values in each column indicate a significant difference ($P < 0.05$, Tukey's method).

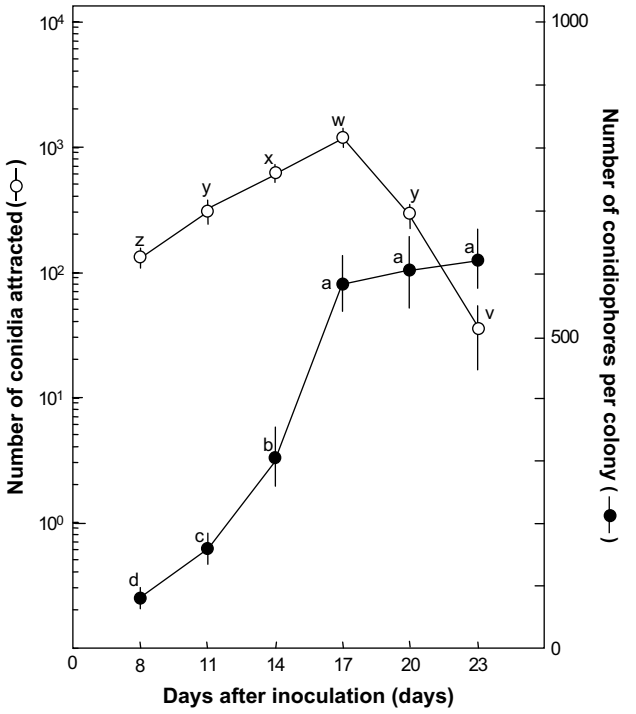

Fig 5 – Numbers of conidiophores per colonies of *Oidium neolycopersici* on tomato leaves and pseudochain conidia collected tridaily from the same colonies. Five colonies on different leaves were used for each tridaily collection of conidia. Data are means and standard deviations of five replications. Standard deviations are presented by error bars on the graph. Different letters indicate a significant difference ($P < 0.05$, Tukey's method).

projected germ tubes downwards, and developed appressoria when the germ tube tips touched the leaf surface (Fig 6B). Almost all appressorium-forming conidia developed haustoria within 48 h after inoculation. Conidia obtained from different aged colonies of inoculated leaves showed similar rates of these infection events, and the time duration of each event was comparable.

The germination rate and infectivity of the conidia that were deposited on the leaf surface are shown in Table 2. Conidia on the leaves were collected by the pipetting method after pseudochain conidia were collected, as the attractive force created by the electrified probe was insufficient to attract all conidia on the leaf surface. The germination rate among conidia on the leaves was less than 20 %, and this rate further decreased as the colonies aged. Approximately 60 % of these conidia germinated upwards, then failed to form appressoria. The conidia germinating downward formed appressoria, followed by successful haustorial formation.

Discussion

Previously we devised an electrostatic attraction method to collect conidia of barley powdery mildew without damaging conidia and conidiophores (Moriura *et al.* 2006a, 2006b). This method can also be applied to safely collect conidia of tomato powdery mildew. In this study, we show direct microscopic evidence that conidia were attracted to the electrified insulator probe and that these attracted conidia germinate successfully. Our results show that this method can obtain an ideal conidial population in which all the conidia germinate simultaneously.

One of the major characteristics in *Oidium neolycopersici* conidial morphogenesis is the formation of pseudochains under different humidities (Kiss *et al.* 2001), wind velocities (Oichi *et al.* 2006), or both (Whipps *et al.* 1998). These pseudochains have varying numbers of constituent conidial cells (Arredondo *et al.* 1996; Kiss *et al.* 2001). In our previous study (Oichi *et al.* 2006), mature conidia remained on conidiophores in conditions of no or low wind velocity, and dropped to the leaf surface when pseudochains reached four conidia in length. Pseudochains comprised of five or more conidial cells were seldom observed on inoculated leaves in greenhouses. The conidia within pseudochains separated from each other and began a new infection cycle, covering the entire leaf surface with superficial secondary hyphae (Oichi *et al.* 2006). However, the conidia subsequently produced from the resulting pseudochains fell on superficial hyphae within the powdery mildew colonies on the leaves, and did not germinate.

Table 1 – Rates of *Oidium neolycopersici* conidia showing abnormal morphological changes in suspensions containing chemical spreading agents (surfactants)[a]

Spreading agent	Incubation time (min)	Dilution				
		10^1	10^2	10^3	10^4	10^5
Mixpower	5	54.7 ± 2.9	43.5 ± 2.4	15.2 ± 1.9	0	0
	30	77.6 ± 1.7	53.8 ± 2.9	33.6 ± 2	0	0
Silwet	5	98.3 ± 0.8	96.4 ± 1.5	50.8 ± 2.4	20.9 ± 1.3	15.9 ± 1
	30	100	100	82.8 ± 2.2	55.2 ± 2.9	23.8 ± 2.6
Submerge	5	99.8 ± 0.4	97.6 ± 1.8	66.6 ± 2.5	54.4 ± 3.6	26.3 ± 3.7
	30	100	99.8 ± 0.4	98.2 ± 1.7	74.8 ± 2.9	32.1 ± 2
Dine	5	53.1 ± 5.5	51.4 ± 2.6	20.4 ± 3.1	9.4 ± 1.4	6.8 ± 1.1
	30	80.8 ± 3	76.5 ± 1.2	37 ± 2.2	14.8 ± 1.9	11.7 ± 1
Mairino	5	86.8 ± 2.1	58.3 ± 2.5	37.3 ± 1.9	26.9 ± 1.1	1.5 ± 0.5
	30	100	72.5 ± 2.2	45.4 ± 3.6	33.5 ± 0.9	16.2 ± 2.4

a Data are means and standard deviations of five replications.

Similar inhibition of conidial germination has been observed among other powdery mildew pathogens when conidia fall within powdery mildew colonies (Carver *et al.* 2001). Our first objective was to determine whether these dormant conidia on leaves would represent an active inoculum after collection. The present electrostatic method made it possible to specifically collect the conidia from conidiophores. Our results indicated that the population of conidia deposited on the leaves became larger as the colony aged, but at the same time the germination rate decreased rapidly.

If conidia can be collected from pseudochains before they fall to the leaf surface, they are an ideal inoculum, as they are fully mature within the pseudochains (Oichi *et al.* 2006). Our results indicated that it takes 4 d for pseudochains to reach maximum length (four-cell chain), as the conidiophores produce mature conidia at their apical tip at approximately 20-h intervals (Oichi *et al.* 2004). This study was undertaken to examine whether the conidia in pseudochains retain a high germination rate while standing erect on conidiophores. We collected conidia exclusively from within pseudochains, without contamination by on-leaf conidia. However, we did not trace their positions within the pseudochains, that is, the age of each constituent conidial cell within each pseudochain. Nevertheless, the population was fully germinative, indicating that the conidia retain their capability to germinate while they remain on conidiophores.

In order to ensure high conidial germination rates, environmental conditions should be optimized. In particular, humidity should be very high (RH 100 % or near) (Whipps & Budge 2000; Jacob *et al.* 2008). We confirmed that conidia germinated successfully in high humidity conditions. Under these conditions, numerous fine water drops were found around the conidia on the probe. These may be a useful water source to initiate conidial germination. From this point of

Table 2 – Infection behaviour of conidia collected from *Oidium neolycopersici* colonies on tomato leaves

Source of conidia	Methods for collecting conidia	Age (days) of colonies	Conidia on leaves[a]									
			Germination (%)		Appressorial formation (%)		100B/A (%)		Haustorial formation (%)		100C/B (%)	
			A		B				C			
Pseudochains	Electrostatic[b]	5	90.9 ± 3.4	x	42.4 ± 2.8	x	46.6 ± 1.1	x	41.1 ± 2.8	x	96.9 ± 3	x
		8	91.3 ± 2.6	x	42.4 ± 2.4	x	46.4 ± 1.1	x	41.2 ± 3.5	x	97.3 ± 1	x
		11	91.7 ± 2.9	x	42 ± 2.8	x	45.8 ± 0.7	x	41.1 ± 2.8	x	98 ± 2	x
		14	91.4 ± 2.4	x	42.5 ± 2.4	x	46.5 ± 0.7	x	42 ± 2.6	x	97.7 ± 1.9	x
		17	89.3 ± 2.5	x	40.6 ± 3.2	x	45.5 ± 0.7	x	39.3 ± 3.5	x	96.7 ± 1.8	x
		20	89.1 ± 1.9	x	39.4 ± 3.1	x	44.8 ± 1.2	x	38.2 ± 2.9	x	96.8 ± 1.4	x
Leaf surfaces	Pipetting[c]	14	13.8 ± 1.7	y	6.3 ± 1.5	y	45.8 ± 3.3	x	5.7 ± 1.9	y	90.2 ± 6.6	x
		17	13.4 ± 1.9	y	6 ± 1.5	y	44.8 ± 2.6	x	5.2 ± 1	y	89.4 ± 7.1	x
		20	11.7 ± 1.3	y	5.7 ± 1.3	y	49.1 ± 4.7	x	5.3 ± 1.2	y	93.6 ± 6.4	x

a Conidia were collected from ten colonies on different leaves and made up to a final density of 10^4 conidia ml^{-1}. Germination, and appressorial and haustorial formation by the conidia inoculated were determined at 6, 14, and 24 hr after inoculation, respectively. Data are means and standard deviations of three replications. Different letters on mean values in each column indicate a significant difference ($P < 0.05$, Tukey's method).

b An electrified probe was used to electrostatically attract pseudochain conidia formed on conidiophores from the same colonies every 3 d. Conidia were suspended in water containing Mixpower (10^4-fold dilution) and inoculated into leaves of one-month-old tomato seedlings.

c After the electrostatic attraction of conidia, the colony was covered with water containing Mixpower, and pipetted several times to suspend conidia deposited on the leaf surface. Different colonies on different leaves were used for each collection.

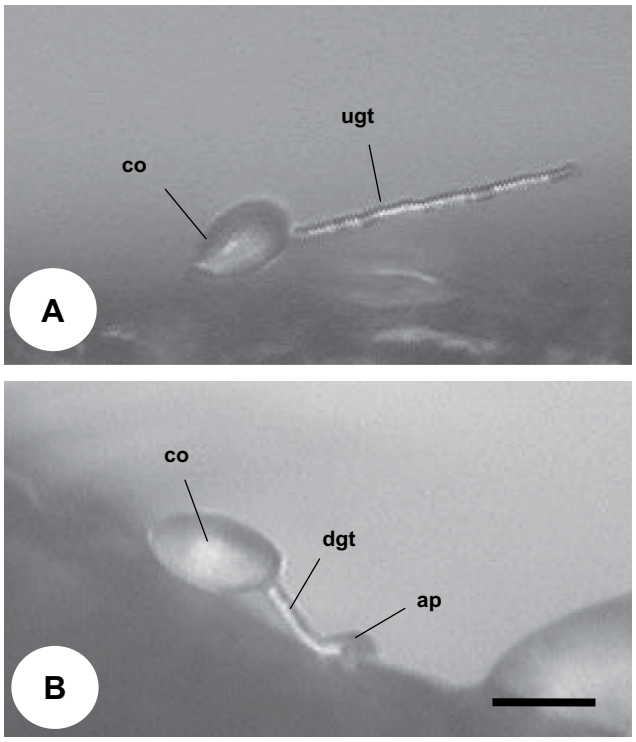

Fig 6 – *Oidium neolycopersici* conidia projecting germ tubes upwards (A) and downwards (B) on tomato leaves 10 h after inoculation. Conidia germinating upwards did not form appressoria at their tips, whereas conidia elongating germ tubes downwards produced appressoria when their germ tube tips touched the leaves. Abbreviations: co, conidium; ugt, germ tube elongating upwards; dgt, germ tube elongating downwards; ap, appressorium. Bar = 10 μm.

view, suspending conidia in water may be useful to provide sufficient moisture for germination. The conidia of *O. neolycopersici* were immiscible with water; however, the addition of a surfactant dispersed conidia uniformly within the suspension. The use of surfactants is popular for suspending powdery mildew conidia in water, but they can be toxic to conidia (Nicot *et al.* 2002). Some surfactants in chemical spreaders were highly toxic to the conidia of *O. neolycopersici*, causing rapid cellular disorganization soon after mixing. To retain the high conidial germination rate, it was essential to select spreaders with no or low toxicity, and to limit the time that conidia were suspended. In fact, Mixpower was used as it was the least toxic (Table 1).

The method described in this study successfully obtained an ideal inoculum of the powdery mildew pathogen, with conidia that germinated simultaneously. However, this does not imply that all germinating conidia successfully establish an infection. Interestingly, more than half of germinating conidia projected their germ tubes upwards so that the germ tube apexes did not touch the leaves. These conidia failed to form appressoria, and thus failed to invade the leaves. Clearly, contact between the leaf and the germ tube apex is an essential event to develop appressoria, because the conidia germinating upwards formed appressoria normally when their germ tube apexes were redirected with a micromanipulator to touch the leaf surface (data not shown). Actually, the requirement of contact between the germ tube and the leaf surface for appressorium development is well documented in other plant pathogens like *Erysiphe pisi* (Fujita *et al.* 2004), *Colletotrichum graminicola* (Apoga *et al.* 2004) and *Magnaporthe grisea* (Xiao *et al.* 1994). Because of this germination characteristic, the highest infection efficiency that was achieved was only 40 % of the germinative conidia, that is, the percentage that elongated germ tubes downwards to touch the leaves. In the previous light microscopic observations of infection behaviour by tomato powdery mildew conidia, inoculated leaves were fixed in an alcoholic lactophenol solution for leaf decolouration and subsequent pathogen staining (Huang *et al.* 1998; Kashimoto *et al.* 2003a, 2003b; Sameshima *et al.* 2004; Miesler-ová *et al.* 2004). However, this conventional method for observation is not suitable for the conidia at the stage before appressorial formation, because conidia are lost while fixing the specimen. The present high-fidelity digital microscope is a useful tool for detecting morphological events by *O. neolycopersici* conidia at the pre-appressorial stage, as this type of the microscope enables detailed observations of conidia on the leaves without fixation (Matsuda *et al.* 2005a).

The attractive force created by the electrified probe is directly proportional to the voltage applied, and inversely proportional to the distance between the probe tip and the leaf (Matsuda *et al.* 2006; Moriura *et al.* 2006b). This relationship applies to the collection of the tomato powdery mildew conidia from leaves (Fig 3). Under the growth conditions described in this study, the pathogen developed approximately 700 conidiophores in single colonies for three weeks, and produced a total of 2700 conidia from these conidiophores (Fig 5). The tridaily collection of pseudochain conidia by the present electrostatic attraction method enabled collection of all conidia from conidiophores before they fell to the leaf surfaces, resulting in a conidial population with a high germination rate. Thus, we established an easy and practical method to obtain a population of simultaneously germinating tomato powdery mildew conidia.

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